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Stress-Wave-Assisted Transport Through the Plasma Membrane In Vitro

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Background and Objective: **Laser-induced stress waves have been shown to alter the permeability of the plasma membrane without affecting cell viability. The aim of the work reported here was to quantify the molecular uptake by cell cultures in vitro and determine optimal stress-wave parameters.**
Study Design/Materials and Methods: **Human peripheral blood mononuclear cells were exposed to laser-induced stress waves in an experimental arrangement that eliminated interference from ancillary effects such as plasma, heat, or cavitation. A radiolabeled compound (tritiated thymidine) was used as the probe.**
Results: **Stress waves enhanced the diffusion of tritiated thymidine by inducing a transient permeabilization of the plasma membrane. Furthermore, maximum intracellular concentration (2×10^5 thymidine molecules/cell or 10% of the extracellular concentration) was reached with only 2–3 stress waves.**
Conclusion: **Laser-induced stress waves provide an efficient method for delivering molecules through the plasma membrane into the cytoplasm of cells. Lasers Surg. Med. 20:216–222, 1997.**

Key words: ablation; cell viability; drug delivery; membrane permeability; shock waves; thymidine

INTRODUCTION

Laser-induced stress waves (LSW) and their effects on cells and tissue have been the subject of numerous studies. This area of research has been reviewed recently by Doukas and Flotte [1]. The development of techniques that enable investigation of the biological effects of LSW without interference from those induced by heat, plasma, or cavitation have facilitated the study of cellular responses caused exclusively by stress waves [2,3]. Cell cultures exposed to stress waves sustain structural and functional injuries that depend on peak stress, rise time, and number of pulses applied [2–4]. The primary target of the stress waves appears to be the plasma membrane. Red Blood cells (RBC) exposed to stress waves, e.g., release hemoglobin into the extracellular medium [2,5,6]. Recently, the study of influx and efflux of membrane impermeable dyes have confirmed that the action of LSW on the cell membrane is to alter its permeability [7–9]. Lee et al. [9] have applied time-resolved imaging to measure the kinetics of the plasma membrane. The membrane permeabilization lasted for a period of 10–80 sec, depending on cell type. Furthermore, if the applied stress was below the threshold for damage, the cells remained viable.

Alteration of the plasma membrane by stress waves is not exclusive to LSW. Holmes et al. [10], Gambhiler et al. [11,12], and Delius et al. [13] have shown that extracorporeal shock waves (ESW) also increased the permeability of the plasma membrane. Both ESW and LSW share

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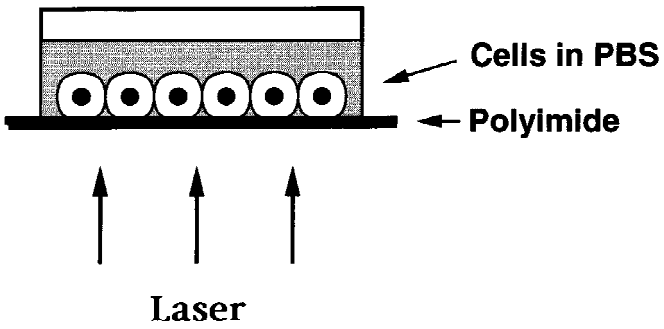

Fig. 1. Experimental arrangement. Target cells (PBMC) were centrifuged to form a monolayer on the polyimide film. A pulse from the excimer laser (ArF) was moderately focused on the polyimide film. Stress waves were produced by ablation of the polyimide and launched into the medium containing the cells.

similar characteristics. They are broadband, unipolar, mostly compressive waves, although tensile components are observed in ESW [14]. In fact, many of the biological effects induced by stress waves generated during laser irradiation have counterparts in the biological effects of ESW. The latter have been reviewed by Coleman and Saunders [15].

In this report we have employed a radiolabeled compound to determine the number of molecules loaded into cells in vitro during the action of LSW. We have shown that LSW provide an efficient method for molecular delivery. Furthermore, maximum intracellular concentration is reached with few pulses.

MATERIALS AND METHODS

The experimental arrangement has been described previously [7]. The cell cultures were placed inside wells drilled on a plexiglas block 1.5 cm thick (Fig. 1). The wells were 3 mm in diameter and were sealed at one end with a polyimide film (300 NH Kapton, Dupont, DE) 75 μm thick. Stress waves were generated by laser ablation of the polyimide film and launched into the medium containing the cells. The laser source was an ArF (193 nm, 200 mJ) excimer laser (Lambda Physik EMG 103MSC, Acton, MA). The laser beam was focused on the polyimide target by an optical system consisting of a spherical (focal length 31 cm) and a cylindrical (focal length 20 cm) lens to a spot size ~3 mm in diameter.

Stress waves were measured at the target site with a polyvinyledene fluoride (PVDF) transducer [16]. A 75-μm polyimide film was placed on the transducer using silicon grease for acoustic contact. An aperture was positioned over the center of the laser beam with the transducer mounted directly behind it. The transducer signal was recorded by a digital oscilloscope (LeCroy 9360, Lecroy Corp., Chestnut Ridge, NY) using 1 MΩ termination. The transducer was calibrated by measuring the signal generated by a known momentum transfer. A light ball bearing was dropped on the transducer, and the impact force was calculated from the conservation of momentum, the mass of the ball bearing, and the time between impacts [17].

Figure 2 shows a stress wave generated during the ablation of polyimide by the ArF laser. The stress wave has a fast rise time (~10 nsec) and a duration of ~80 nsec. The measured stress was first corrected for the acoustic impedance difference between the polyimide and the transducer to determine the stress applied to the polyimide. The peak stress applied to cell cultures in the medium was estimated from the acoustic impedances of the polyimide ($Z_p = 3.1$ MPa m^{-1} s) and water ($Z_w = 1.5$ MPa m^{-1} s) using the expression $2Z_p/(Z_p + Z_w)$. A peak stress of 380±40 bar was used in the experiments described here.

Target cells were placed inside the wells and centrifuged to form a monolayer. The wells were placed at the position of the aperture and irradiated. The large aspect ratio of the sample (beam size/sample thickness >300) insured that the cells were exposed to planar waves. In previous studies [2,3], we have shown that the presence of the polyimide film eliminates the effects of plasma, heat, and UV radiation. Although we have been unable to prove or disprove directly the presence of cavitation, we have shown indirectly that cavitation was not generated at peak stress of up to 650 bar [3], a much higher stress than presently used.

Cell Preparation

Human peripheral blood mononuclear cells (PBMC) were used as target cells. Blood was drawn in a heparinized syringe from healthy human volunteers. The blood was mixed with Dulbecco's phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}. The blood suspension was carefully layered on to a ficoll-hypaque gradient in a 50-mL centrifuge tube. The tube was spun at 1,200 RPM (200 g) for 40 min. The PBMC at the gradient/supernatant interface were collected and washed three times with PBS. The cells were adjusted to a concentration of 8×10^6/mL in PBS. After the third wash, the PBMC were split into

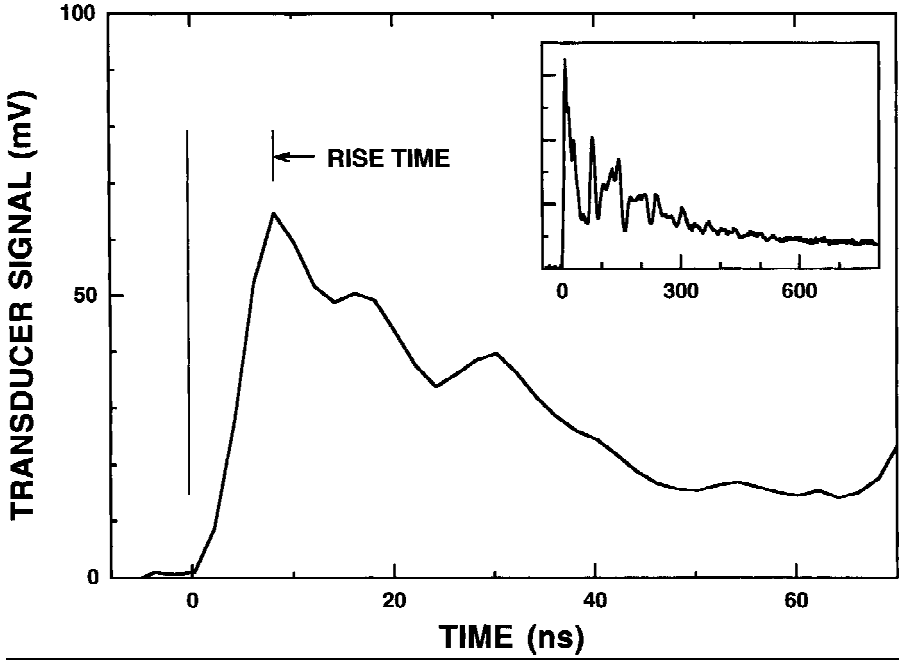

Fig. 2. Waveform of a stress wave generated by ablation of polyimide film 75 mm thick by 193-nm radiation. The stress wave was measured by a calibrated PVDF transducer. The leading edge (0–100%) of the stress wave is ~10 nsec and the duration ~80 nsec. The combined temporal resolution of the transducer oscilloscope is ~5 nsec. The insert shows the extended waveform of the stress wave. The secondary peaks shown are the reflected waves at the polyimide-transducer interface.

two equal portions. Half the cells were resuspended in PBS to which a concentrated solution of dipyridamole (DPM) (Sigma, St. Louis, MO) in ethanol was added to achieve a final concentration of 2.5 μM. The other half of the cells was resuspended in PBS only to which the same volume of ethanol, as in the first portion, was added. All cells were incubated at room temperature (20°C) for 20 min. After incubation, enough tritiated thymidine (sp. ac 6.7 Ci/mmol, New England Nuclear, Boston, MA) was added to each cell suspension to achieve a concentration of 15 μM. A 50-μL aliquot of either cell suspension was then placed into each well. Subsequently, the plate was spun for 5 min at 500 RPM at room temperature in order to insure that the cells were in contact with the polyimide at the bottom of the well.

Application of Laser-induced Stress Waves

The cell cultures were exposed to excimer laser-induced stress waves at a repetition rate of 0.1 Hz. The low repetition rate used in the present experiments was chosen in order to take advantage of the long resealing time of the plasma membrane [9]. In a number of control experiments, the cells on the polyimide film during and immediately following the application of stress waves were counted to insure that there was no loss of cells during the procedure.

Oil Stop Technique Using 1-Bromododecane

After irradiation, the cells from two wells at a time were removed and carefully placed on top of 600 μL of 1-bromododecane in a 1.5 mL microcentrifuge tube, as described by Wohlhueter et al. [18]. The tubes were spun at 13,000 g for 1 min. After centrifugation, the supernatant was removed leaving only the PBMC pellet in the microcentrifuge tube. To each microcentrifuge tube, 100 μL of distilled water were added. The cells in the microcentrifuge tubes were then disrupted using a sonicator (Cole Palmer 8852, Fisher Scientific, Springfield, NJ). After sonication the contents of each microcentrifuge tube were placed into a scintillation vial containing 2.0 mL of scintillation cocktail (Beckman Ready Gel, Fisher Scientific). The radioactivity of each vial was measured using a Beckman scintillation counter, model LS 3801. The mean of the radioactive counts per min (cpm) of the samples for each condition was calculated. The data from many experiments have been combined, each data point rep-

resenting an average of at least 12 wells. The error bars in the figures are the standard deviations. The background level of the scintillation counter was 30 cpm.

The measured counts per min were converted to number of thymidine molecules. The conversion factor was calculated as follows. Different concentrations of tritiated thymidine, obtained by serial dilution of a stock solution, were used for calibration of the scintillation counter. The number of thymidine molecules in a vial was calculated from the concentration and the volume of the solution measured. A linear regression of the number of thymidine molecules in the vials vs. the measured radioactivity was used to calculate the conversion factor. Addition of lysed cells in the thymidine solution decreased the number of counts by ~8%. This was probably caused by the absorption of β particles by cellular proteins. The conversion factor, corrected for the presence of cells in the vials, is 4×10^7 molecules/cpm.

Propidium Iodide/Fluorescein Diacetate Assay

Fluorescein diacetate (FDA), a vital stain, was added to suspensions of cells to achieve a final concentration of 5 μg/mL. Propidium Iodide (PI), also a vital stain, was added to cell suspensions to make a final concentration of 16 μg/mL. The cells were examined under an epiluminescent fluorescence inverted microscope (IM35, Zeiss, Oberkochen, Germany) 3 min after adding the vital stains.

Tritiated Thymidine Incorporation Assay

In separate experiments, proliferation of PBMC exposed to stress waves was assayed by the incorporation of tritiated thymidine. Test PBMC were exposed to stress waves and control PBMC were treated in an identical fashion except they were not exposed to stress waves. All PBMC cultures were then stimulated for 72 hr with mitogen by the addition of 1.25 μg/mL of phytohemagglutilin (PHA) (Ha 17, Burroughs Wellcome, Beckenham, UK). PBMC were suspended in RPMI 1640 (GIBCO) with 20% fetal bovine serum (GIBCO) and antibiotics (penicillin 200 IU/mL and streptomycin 200 μg/mL). The PBMC cultures were incubated at 37°C in an atmosphere of 5% CO_2. The PBMC were subsequently centrifuged and resuspended in complete medium with 0.25 μCi of tritiated thymidine and plated in a 96-well, flatbottom microliter plate. The plate was incubated for 4 hr. The cells were then disrupted; the contents were collected on glass fiber filter strips and washed freely using an automated harvester (MASH II, Microbiological Associates, Walkersville, MD). The dried filter papers were suspended in scintillation fluid and the radioactivity measured using the Beckman scintillation counter.

RESULTS

Tritiated thymidine provides a simple and inexpensive way to investigate and quantify the uptake of molecules by cells exposed to stress waves because the measured radioactivity is directly related to the number of thymidine molecules in the cells. Although thymidine is hydrophilic (the octanol/aqueous buffer partition coefficient is 0.01) and diffuses very slowly through the plasma membrane, the majority of mammalian cells contain nonconcentrative nucleoside transporters [19]. The presence of nucleoside transporters complicates the interpretation of the results because stress-wave-induced thymidine uptake is additive to thymidine actively transported into cells. There are, however, potent inhibitors of nucleoside transport, such as dipyridamole, dilazep, and lidoflazine, that greatly reduce the activity of nucleoside transporters [19].

Thymidine is not a component of the primary pathway for DNA synthesis, but is introduced via phosphorylation. Furthermore, thymidine may be degraded by a number of other pathways [20]. The main consideration for the studies presented here was the possible degradation of thymidine and subsequent efflux of radioactive products during the experiment, which would result in a low estimate of intracellular concentration. However, once the cells were centrifuged through 1-bromododecane, tritiated thymidine remained in the pellet. In several experiments the time between the application of stress waves and the oil stop procedure was reduced to <30 sec. There was no difference in measured radioactivity. We conclude, therefore, that if there was any efflux of thymidine during the experiment, it was negligible. It should be pointed out that the PBMC were unstimulated, i.e., in resting phase. Thymidine incorporation into DNA during the experiment was below detection level.

Figure 3a shows the uptake of tritiated thymidine by PBMC cultures exposed to five stress waves. Tritiated thymidine at 15 μM extracellular concentration was present during the application of stress waves. Cells were treated with or without DPM (±DPM) and were either exposed or

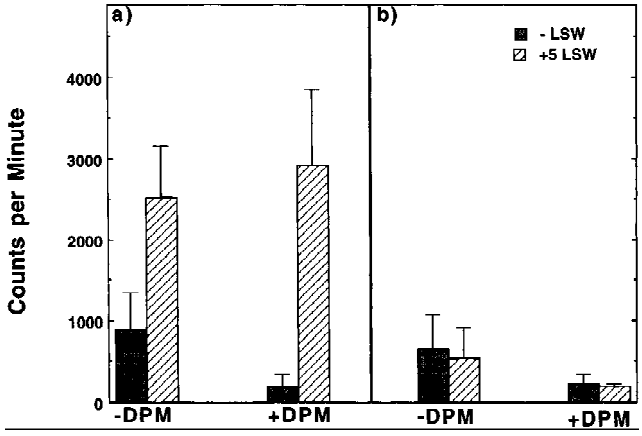

Fig. 3. PBMC cultures were treated with or without DPM (±DPM) and were either exposed or not exposed to five LSW (±LSW). The extracellular tritiated thymidine concentration was 15 mM in all cases. Thymidine uptake was measured in a scintillation counter as described in the text. (**a**) Tritiated thymidine was present during exposure to stress waves. The effect of stress waves on the cells was to increase the thymidine uptake with or without DPM. Incubation of the cell cultures with DPM greatly reduced the activity of the thymidine transporters. (**b**) Tritiated thymidine was added to the medium 15 min after exposure to stress waves. No effect was observed in all cases. Tritiated thymidine has to be present during the application of stress waves in order to diffuse into the cytoplasm.

not exposed to 5 LSW (±LSW). There are four combinations, all of which were tested. The effect of LSW on the cells was to increase the thymidine upake. Incubation of the cell cultures with DPM greatly reduced the activity of the transporters so that a more accurate value of the thymidine uptake under the action the stress waves could be obtained. Note that the number of thymidine molecules inside the cells after five stress waves is the same in both the treated with DPM and the untreated cell cultures. The thymidine intracellular concentration appears to have reached saturation. When tritiated thymidine was added 15 min after the application of stress waves (Fig. 3b), thymidine uptake did not increase. Furthermore, the stress waves did not seem to modify the inhibitory action of DPM.

Figure 4 shows the measured radioactivity (left scale) as a function of the number of pulses applied to cells. From the measured radioactivity and the conversion factor, the number of thymidine molecules taken up by the cells was derived. This number divided by the total number of cells gave the average number of thymidine molecules per cell (right scale). The salient feature is that the maximum number of thymidine molecules taken up by the cells was reached after only two stress waves. The difference in the number of molecules taken up by two, three, or five stress waves is not statistically significant.

Cell cultures were assayed for viability using PI/FDA assay. In addition, cell size distribution was measured in a Multisizer Coulter Counter. The viability of the cell cultures was >93% in all experiments. Furthermore, the size distribution of cell cultures exposed to stress waves showed no discernible difference from that of the controls.

Cell proliferation was investigated in separate experiments. Cell cultures exposed to stress waves as well as controls (cell cultures underwent identical procedures but not subjected to stress waves) were PHA-stimulated for 72 hr and subsequently were assayed for incorporation of tritiated thymidine. There was no difference in the incorporation of thymidine between the two groups, indicating that exposure of cell cultures to stress waves did not have any effect on mitogen stimulation and subsequent cell proliferation.

DISCUSSION

Research performed over the past 3 years has unequivocally shown that LSW alter the permeability of the plasma membrane of cells in vitro. Molecules present in the extracellular matrix can diffuse into the cytoplasm due to the concentration gradient before the plasma membrane reseals after a few seconds. The resealing time appears to be a property of the cell type. Furthermore, if the peak stress applied is below the threshold for cell injury, the cells survive.

Assuming that all cells in the culture are equally loaded, an average of 2×10^5 molecules of thymidine are delivered into each cell under the present experimental conditions. It should be noted that not all the internal volume of the cells may be accessible to thymidine molecules. The observations of Brown and Berlin [21] suggest that 40–50% of the cytosolic volume is occupied by microtubules that form a mechanically irreducible space. In addition, organelles (e.g., mitochondria, lysosomes, and golgi) may further reduce the cytosolic volume available to thymidine. From the size distribution of the cells, we can estimate the total volume of the cell culture, assuming that the cells are spherical and that the total volume of the cells is accessible to thymidine molecules. The average intracellular concentration reached with two pulses and 15 μM extracellular

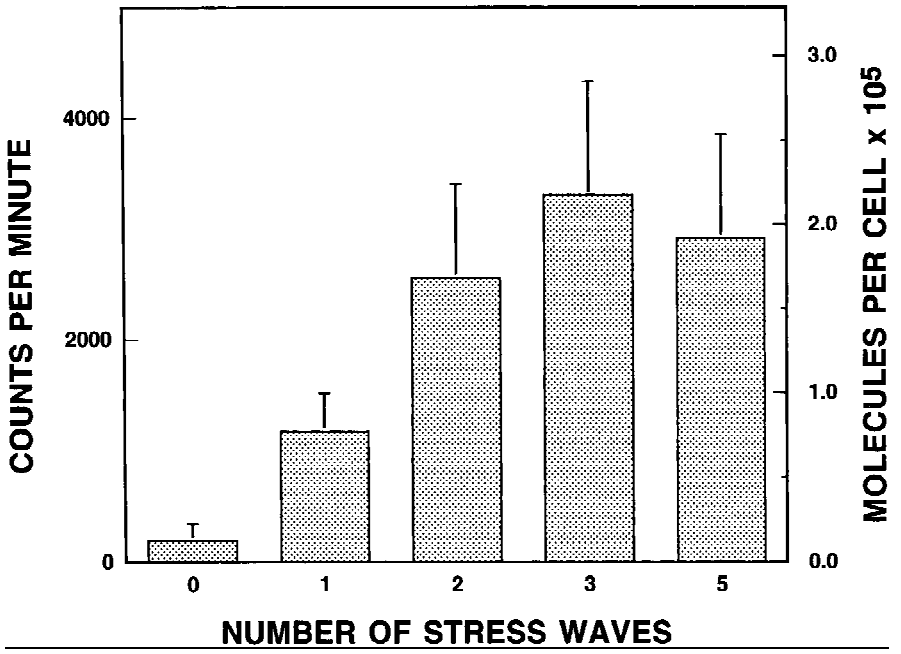

Fig. 4. The average number of intracellular thymidine molecules per cell vs. the number of applied stress waves. Control cultures were not exposed to stress waves but otherwise were subjected to identical procedures. All cells were treated with DPM prior to the application of the stress waves. The number of molecules taken up reached saturation with two stress waves. The difference in the effect of two, three, and five stress waves is not statistically significant. However, even a single stress wave induces a statistically significant increase of the intracellular concentration of thymidine ($P<1.5\ 10^{-6}$).

concentration of thymidine is 2 μM, or ~10% of the extracellular concentration.

The capability of stress waves to deliver molecules into cells in vitro has been observed in other cell lines. This capability to deliver large molecules into cells with no effect on cell viability may be an efficient method for drug delivery. Flotte et al. [8] showed that stress waves facilitated the introduction of β-galactosidase gene into the COS cell line. They also observed that the gene subsequently was expressed in a significant fraction of the transfected cells.

The increased permeability of the plasma membrane caused by stress waves can explain the enhancement of the cytotoxicity of chemotherapeutic drugs when present in the extracellular matrix during the application of stress waves. Flotte et al. [7] have shown that stress waves enhanced the cytotoxicity of a number of compounds, including cis-platin, present in the extracellular medium. EMT-6 cell cultures exposed to LSW showed decreased viability only when the compounds were present in the medium during the application of the stress waves. Addition of the compounds to the medium after the stress waves were applied had no effect. Our present understanding of the synergism of drugs and stress waves is that stress waves induce a transient increase of the membrane permeability that results in the diffusion of drug molecules present in the medium into the cells increasing the intracellular concentration above the toxicity level. The enhancement of drug cytotoxicity under the influence of stress waves has also been investigated with ESW. A number of studies in vitro as well as in vivo have shown that stress waves can potentiate chemotherapeutic drugs such as cisplatin [22–24], doxorubicin [25], and adriamycin [26].

The alteration of the membrane permeability resembles, to some extent, that induced by electroporation [27]. However, the physical attributes of stress waves and high voltage pulses, as used in electroporation, are substantially different to preclude a common mechanism for the permeabilization of the plasma membrane. Nor is hydrostatic pressure relevant to the interpretation of the effects of stress waves. Although hydrostatic pressure has been reported to increase membrane permeability [28], the presence of strong stress gradients is essential for membrane permeabilization.

The mechanisms involved in the permeabil-

ization and recovery of the plasma membrane are not known. We know, however, that the plasma membrane permeabilizes in <60 msec and recovers in 10–80 sec [9]. The disparity in the time scales of the permeabilization and recovery of the membrane suggests that the two processes are driven by different mechanisms.

In conclusion, laser-induced stress waves appear to be effective in altering the permeability of the plasma membrane allowing molecules present in the extracellular matrix to diffuse into cells.

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